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REPRODUCIBLE HIGH QUALITY RNA ISOLATION FROM POLYPHENOL-, POLYSACCHARIDE AND PROTEIN-RICH TISSUES OF A ORPHAN LEGUME CROP BLACKGRAM [*VIGNA. MUNGO* (L.) HEPPER]

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ABSTRACT

Blackgram (*Vigna mungo* (L.) *hepper*), is one of the important pulse crops in the world especially in India, it is cultivated both in Kharif and Rabi seasons under semi irrigated and dry land conditions, where the drought stress is a major problem. Plants adapt alternate pathways for their existence during the periods of stress, especially at flowering and pod filling stages. It is evident that resistance or sensitivity to the stress depends on species, genotypes and even developmental stages and it is essential to identify novel stress responsive genes from the resistant species to develop the stress tolerant plants. To understand the gene expression in response to the drought stress the isolation of RNA is a preliminary step. Isolation of RNA in pure form involves interference of substances like polyphenols, secondary metabolites and polysaccharides which can co precipitate and making problems to get pure RNA. Use of earlier methods such as combine guanidine and CTAB, SDS and phenol not successful in getting good quality and quantity RNA yield from blackgram. There are no previous reports available in the literature regarding isolation of RNA from drought stress induced blackgram. In our study an attempt was made to isolate the RNA from flowers and roots of blackgram. We have used sucrose- sodium chloride extraction buffer to isolate the RNA and in the final step lithium chloride ($LiCl_2$) was used to selectively precipitate the RNA. Using this protocol we have successfully obtained the high quality total RNA from leaves, flowers and roots of black gram which will be used further for cDNA library construction and gene expression studies using real-time PCR, RACE, stress expression studies and genetic diversity studies.

KEYWORDS: Lithium Chloride, RNA, Drought Stress and DEPC, RT-PCR

INTRODUCTION

Blackgram [*Vigna. mungo* (L.) Hepper] is an important legume crop widely cultivated in Asia for the purpose of protein, amino acids, vitamins, and minerals and for its plant parts as fodder and green manure. This legume crop adapts well to various cropping systems owing to its ability to fix atmospheric nitrogen (N_2) in symbiosis with soil bacteria of *Rhizobium* spp., apart from having rapid growth and early maturity. Recent statistical evidences have revealed that blackgram yields depleted year by year in India, particularly in Andhra Pradesh (Directorate of Economics and Statistics, Department of Agriculture and cooperation, Ministry of Agriculture, Government of India, 2012). Even though blackgram is a good source of protein, this crop is neglected in breeding research, both at national and international levels, particularly in the field of genomics. Blackgram is classified into the genus *Vigna*, subgenus *Ceratotropis* (known as Asian *Vigna* or Asiatic gram), section *Ceratotropis* and Spp. mungo (Tomooka *et al.,* 2002). It is diploid in nature with 2n=2x=22 having

small genome size estimated to be 0.59 pg/1C (574 Mbp) (Arumuganathan K and Earle ED, 1991). In terms of genomic resources, blackgram has relatively limited available data, in comparison to other Fabaceae species. A total of 631 EST sequences and 242 nucleotide sequences in blackgram are present in the public domain as of 17 December, 2013 as compared to 44,997 ESTs; 51,511 GSSs in chickpea and 24,176 ESTs; 89307 GSSs in pigeonpea including the whole genome sequence. There is a significant need to improve the functional genomics resources of blackgram to identify the novel genes and to facilitate the research in the area of genomics and molecular assisted breeding for drought stress tolerance.

Isolation of high-quality RNA is a critical step in the successful implementation of functional genomics experiments (e.g., reverse transcription– polymerase chain reaction [RT-PCR], generation of expressed sequence tags, microarray analysis). Generally, all legumes, including blackgram are complicated by the presence of high concentrations of intrinsic polysaccharides, polyphenols, and other secondary metabolites. It was observed that the tendency of increasing concentration of these compounds in plants under biotic and abiotic stresses, such as pathogen infection or drought (Chaves *et al.,* 2003; Logemann *et al.,* 1987; Lopez *et al.,* 1992). Polyphenols present in the tissues become oxidized to quinines and covalently bind to nucleic acids (Loomis, 1974) and polysaccharides coprecipitate with RNA because of having similar physicochemical properties of RNA (Sharma *et al*., 2003; Key *et al*., 2012). Besides this polysaccharide concentration hinders resuspension of precipitated RNA and interferes with absorbance based quantification, enzymatic manipulations and electrophoretic migration (Wilkins and Smart, 1996). If these metabolites are not removed during the process of RNA extraction, they may interfere with highly sensitive downstream applications such as sequence expressed tag marker assisted polymorphism, cDNA library construction, and microarray hybridization or they may completely degrade RNA.

There are many methods available for the isolation of RNA from different tissues. The improved methods like AGPC method (ChomcZynsky and Sacchi, 1987), benzyl chloride method (Suzuki *et al*., 2001), SDS and Phenol method (Thompson *et al*., 1983; Alemzadeh *et al*., 2005) produce high quality RNA from specific plant species. But these methods are time consuming, more laborious and involves toxic chemicals like guanidian thiocyanate, benzyl chloride and CTAB.

Further, there are no reports are available regarding the purity and quantity of extracted RNA from blackgram cultivars both in stressed and unstressed conditions. Thus, there is an immediate need to improve and standardize methods for RNA isolation in blackgram genotypes which can be useful for further biotechnological and genetic engineering studies.

RNA isolation from legume tissues like leaf and seeds has been reported earlier (Datta *et al.,* 1989; Kansal *et al*., 2008) using detergents such as sodium dodecyl sulphate (SDS), followed by lithium chloride (LiCl), but the efficiency of these methods in isolating RNA from different plant parts of blackgram not known. Hence the same method was used to extract pure RNA with slight modifications. This modified method overcomes the difficulties in extracting pure RNA from leaves, roots and flowers of blackgram which are rich in protein, lipid and phenolic compounds and simultaneously avoids the use of toxic chemicals. The isolated RNA from all the tissues is amenable for downstream applications such as PCR, qRT-PCR and cDNA library construction.

MATERIALS AND METHODS

Plant Material, Sampling and Chemicals

A developed variety of blackgram genotype LBG 748 was obtained from Regional Agricultural Research Station, Lam, Guntur. Earthen Pots of 25 cm × 30 cm size were filled with black loamy soil. A total of three seeds for pot were maintained and the plants were irrigated regularly. At 40 DAS plants were kept for drought treatment for about six days. Immediately after treatment the leaves, flowers and roots were collected from the control plants whereas only leaves were collected in case of water stressed plants then plants were reirrigated and kept for recovery. After six days of recovery again leaves were collected from the treated plants. Plant tissues were flash frozen in liquid nitrogen immediately after collection and preserved at -80°C until further studies. The chemicals used were of molecular biology grade and solutions were prepared by using Millipore water. Consumables were of RNase free, plastic ware and pipette tips were treated overnight with 0.1% DEPC-treated distilled water and autoclaved. Mortars and pestles were baked overnight at 140°C.

RNA Isolation

About 1.5 g of plant material (leaf, root and flower) was made to a fine powder by using precooled pestle and mortar and liquid nitrogen. To the powdered plant tissue sample a cocktail of 10 ml of extraction buffer (0.1 M Tris HCl, pH 9.0, 0.25 M Sucrose, 0.2 M NaCl and 10 mM $MgCl_2$) was added in order to extract the entire RNA from all the tissue material. Later 10 ml of freshly prepared 1:1 (v/v) phenol (water saturated), chloroform mixture was added to denature the protein contaminants. This is followed by an addition of 1 ml of 0.5 M potassium EDTA and 1 ml of 20% SDS sequentially and the grinding process was continued. The grinded solution of RNA mixture was transferred to sterile oakridge tubes. To these oakridge tubes containing RNA mixture 144 µl of β – mercaptoethanol was added and shook at 4°C for about 20 min followed by a centrifugation at 16,000 rpm for about 30 min then the supernatant was transferred to another sterile oakridge tube. Equal volume of chloroform and isoamyl alcohol (49:1) was added to this aqueous phase, followed by a brief vortex. Now the contents were spun at 15, 000 rpm for 15 min at 4°C. The aqueous phase formed was transferred to another clean oakridge tube and to this 8 M of $LiCl_2$ was added to bring a final concentration of 3 M and the tubes were preserved at 4°C for about 20 hrs. Later the tubes were centrifuged at 16, 000 rpm for 30 min at 4°C. The supernatant was discarded and precipitate was washed with 5 ml of 2 M $LiCl_2$ followed by 5 ml of 75% ethanol by centrifuging at 15, 000 rpm for 20 min at 4°C. The pellet was dried and dissolved in 150 µl of DEPC water.

Quantification and Integration of Total RNA

The quantity and quality of the RNA obtained was assessed spectrophotometrically at 230, 260 and 280 nm. An aliquot of (1 µl) of the supernatant was used for the quantification of yielded RNA and the absorbance was measured at *A*260 nm. The *A*260/230 and *A*260/280 ratio was used to detect the carbohydrates and proteins contamination. Quality and integrity of isolated RNA was verified by electrophoresing RNA on 1.5% agarose gel and staining with ethidium bromide (Sambrook *et al.,* 1989). The bands were visualised and photographed using Gel documentation unit.

cDNA Synthesis, RT-PCR and Library Construction

All RNA samples were treated with RNase-free DNase (Fermentas), according to the manufacturer's instructions. First strand cDNA was synthesized by reverse transcribing 2 µg of total RNA with RevertAid First Strand cDNA Synthesis Ki (Fermentas) in a 20 µl reaction using oligo $(dT)_{18}$ primers and RevertAid™ M-MuLV Reverse Transcriptase according to manufacturer's instructions. Reverse transcription was performed at 42°C for 1 hour followed by 70°C for 5 min.

All cDNA were stored at −20°C until use.

The cDNAs were PCR amplified (with expected amplicon of 150 bp) by *dhn1* gene (NCBI accession No AY547295) specific primers. PCR reactions were carried out in a final volume of 25 µL reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200µM dNTP, 0.2µl (10µM) of each primer (forward 5'-CACACAGAGATGATGGGTATGG- 3' and reverse 5'- CACAGAGATGATGGGTATGG – 3') and 0.5 U of Taq DNA polymerase. The temperature profile was as follows. The initial denaturation was carried out at 94°C for 2 min later on up to 30 cycles at 94°C for 1 min, 55°C for 1min, and 72°C for 1 min followed by a final extension for 7 min at 72 °C. The cDNA library was constructed with ZAP-cDNA Synthesis Kit (Agilent Technologies USA) using 5 µg of isolated RNA. The library was subsequently used for identification of drought stress responsive genes (unpublished data).

Quantitative RT-PCR Analysis (qRT-PCR)

qRT-PCR was performed on a Lightcycler 480 (Roche) with LightCycler®480 SYBR Green I Master (Roche). The reactions were performed according to the manufacturer's instructions. The PCR program was initiated at 95°C for 5 min to activate *Taq* DNA polymerase, followed by 35 thermal cycles for 10 seconds at 95°C, 10 seconds at 55°C and 15 seconds at 72°C. Melting curve analysis was performed immediately after the real-time PCR. The temperature range used for the melting curve generation was from 55°C to 95°C. All assays were performed using three technical and biological replicates, a non-template control and a non-RT control. The determination of the crossing amplification point (Cp) as well as the relative quantification analysis (ΔΔCT-method) was performed using the Lightcycler 480 software 1.5. The amplification of non-template controls generated Cp values of above 45 and was not detectable. The non-normalized expression data was analyzed by geNorm v3.5 and Norm Finder version 2 whereas the raw Cp values were imported into BestKeeper version 1.

RESULTS AND DISCUSSIONS

Black gram tissues are rich source of protein and amino acids like methionine, tryptophan and lysine. It also contains 55-60% soluble sugars, fibers, starch and unavailable carbohydrates. All these molecules may interrupt the RNA extraction processes and results in poor yield of RNA with degradation. Hence, standard method such as guanidinium-phenol-chloroform method for RNA extraction (Chomezynski and Sacchi 1987) could not be useful for blackgram tissues.

In this study a phenol- based, guanidinium salt-free protocol was developed using the simple low cost chemicals sucrose and sodium chloride. All the reagents used in this protocol were selected based on their properties. Sucrose and sodium chloride were useful to maintain the osmotic pressure, while the Tris buffer maintains the cells at pH 9.0. EDTA binds divalent cations in the lipid bilayer, thus weakening the cell envelope. Following cell lysis, EDTA limits RNA degradation by binding Mg^{+2} ions which is a necessary cofactor for nucleases such as DNase and RNase (Dawson *et al.*, 1986). Addition of high concentrations of β-mercaptoethanol preserves the RNA by preventing production of quinones from phenolics (Venkatachalam, 1999). Moreover the β-mercaptoethanol along with phenol/chloroform might inactivate RNAase by dissociating the disulfide linkages (Venkatachalam *et al*., 1999, Dawson *et al*., 1986, Wang *et al*., 2005).

The SDS disrupts protein–nucleic acid interactions and, together with the chloroform and phenol, denatures the proteins so that they become insoluble in the aqueous solution. The charged nucleic acids will remain in the salty aqueous phase, whereas the proteins, with their aromatic and aliphatic side chains, will partition to the organic phase.

Because phenol: chloroform is immiscible with water, centrifugation results in the formation of two phases, a lower organic phase that contains denatured proteins and an upper aqueous phase that contains nucleic acid. The isoamyl alcohol facilitates the separation of the two phases. Removing the aqueous phase recovers the RNA from the protein, and a second organic extraction with chloroform removes the considerable amount (~6% by volume) of phenol that dissolves in the aqueous phase with the RNA and that can contaminate the RNA. Higher centrifugation speed (g) was maintained for proper segregation of nucleic acids from proteins and polysaccharides (Salzman *et al*., 1999).

To prevent the chemical reaction between polyphenols, polysaccharides and RNA during the extraction the samples were incubated at low temperatures from the first step itself. It is contemplated that low temperatures decreases rate of chemical reactions. As a result, the phenolic compounds did not react with nucleic acids and were precipitated with other debris after the first centrifugation and the supernatant appeared was completely clear.

LiCl has been frequently used to precipitate RNA, although precipitation with alcohol and a monovalent cation such as sodium or ammonium ion is much more widely used. LiCl precipitation offers major advantages over other RNA precipitation methods where they do not efficiently precipitate DNA, proteins and carbohydrates (Barlow *et al.*, 1963). It is the method of choice for removing inhibitors of translation or cDNA synthesis from RNA preparations (Cathala *et al.*, 1983). It also proved to be a simple and rapid method for recovering RNA from in vitro transcription reactions. Ethyl alcohol solution effectively eliminate the salt residues present in the sample (Hidayah Jamalnasir, 2013), there by producing the qualitative RNA. The precipitated RNA was free from contaminating DNA and therefore eliminated the need for decarboxynucleases (DNase).

Using the current protocol total RNA was isolated from the different tissues types, genotypes and drought stressed samples of blackgram. A total of 360µg of RNA was obtained from the 1 gm of leaf sample followed by 280µg from flowers and 230µg from roots. Drought stressed leaf samples yielded 330µg of total RNA. A260/A280 and A260/A230 ratios of the RNA extracts were as high as 1.87, 2.36 respectively, which indicates the presence of low amounts of protein, polysaccharides and polyphenols contamination (Table 1). Purity of RNA samples (A260/A230 ratio) isolated from leaves was 2.36, while in case of root tissue, floral tissue and drought stressed leaves it was 2.1 and 2.21.

The quality of the RNA assayed using the formaldehyde agarose gel electrophoresis. All the RNA samples showed discrete bands corresponding to 28S and 18S rRNA (Figure 1 and 2). The bands were significantly prominent in leaves followed by flowers and roots. Using this protocol high quality of RNA was isolated from the different genotypes of the blackgram. Hence, the above described method is a genotypic independent.

Table 1: Total RNA Yield Isolated from Different Tissues of Blackgram Using $LiCl_2$ Method

Plant Tissues	RNA Yield (µg/g Tissue)	RNA Purity	
		A260/A280	A260/A230
Leaf	360±15	1.875±0.011	2.36±0.014
Flowers	280±30	1.8±0.01	2.1±0.02
Roots	230±10	1.7±0.02	2.1±0.024
Drought Stress Leaf	330±15	1.8±0.01	2.21±0.01

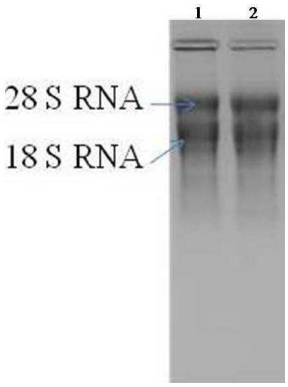

Figure 1: Isolated RNA Showing Intact mRNA

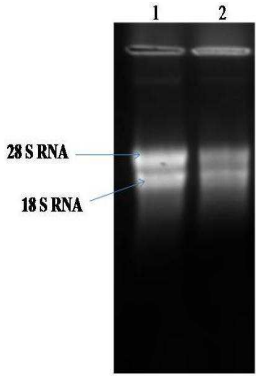

Figure 2: The Pure and Intact mRNA in Flower and Root Samples

1. Control Leaf
2. Drought Stress Leaf

After isolating the high quality total RNA from leaves, flowers, roots and drought stressed samples, cDNA was prepared by reverse transcription. Further, this single stand cDNA was used as template for RT-PCR, qRT-PCR and cDNA library construction. Using the gene specific primers of actin gene (NCBI Acc No FR839671) an expected amplicon of 350 bp was successfully amplified from all RNA preparations indicating the high quality of RNA and acquiescent for downstream applications (Figure 3).

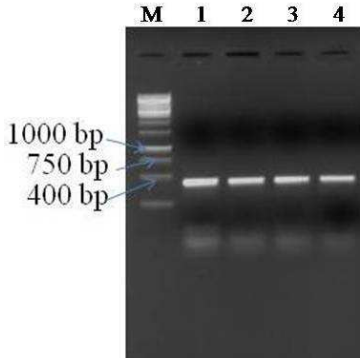

Figure 3: Actin Amplification Products of cDNA

1. Leaf
2. Drought stress leaf
3. Flower
4. Root

CONCLUDING REMARKS

Blackgram is an annual pulse crop and contains large amounts of polyphenols and polysaccharides. Because of these compounds isolation of good quality RNA from different plant parts of blackgram is difficult. Most of the published protocols failed to yield sufficient quantity of high quality RNA from various blackgram tissues suitable for gene expression studies. Our refined protocol with the inclusion of EDTA, NaCl extraction buffer along with an addition of $LiCl_2$ in final step yielded good quality and quantity of RNA, from all the plant parts of blackgram. Intact RNA, higher amount of mRNA recovery, consistent cDNA profile through Differential Display RT-PCR, amplification of higher number of subtracted cDNA transcripts through subtractive hybridization and RT-PCR using gene specific primer confirmed the quality of RNA. Moreover this protocol is simple, fast and inexpensive to perform and allowing efficient

extraction of high-quality RNA from lentil crops, where macromolecular contamination is a major barrier. Hence, this protocol will be useful for isolating high quality RNA suitable for gene expression studies and also for Northern blotting, nuclease protection assays, RNA mapping, *in vitro* translation, RACE and cDNA library construction.

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REFERENCES

1. Tomooka, N, Duncan, A. V., Helen, M., & Nigel, M. (2002). The Asian Vigna: Genus *Vigna* subgenus *Ceratotropis* genetic resources. Kluwer Academic Publishers. P.O. Box 17, 3300 AA Dordrecht, The Netherlands.
2. Arumuganathan, K., & Earle, E. D. (1991). Nuclear DNA content of some important plant species. Plant Molecular Biology Reports, 9 (3): 208-218.
3. Chaves, M. M., Maroco, J. P., & Periera, J. S. (2003). Understanding plant responses to drought from genes to the whole plant. Functional Plant Biology 30: 239-264.
4. Logemann, J., Schell, J., & Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. Annals of Biochemistry, 163: 16-20.
5. Lopez, R., & Gomez, M. A. (1992). A method for extracting intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. Horticulture Science, 27(5): 440-442.
6. Loomis, W. D. (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods in Enzymology, 31: 528-545.
7. Sharma, A.D., Gill, P. K., Singh, P. (2003). RNA isolation from plant tissues rich in polysaccharides. Annals of Biochemistry, 15, 314(2): 319-21.
8. Ky, H., Yeap, S. K., & Napis, S. B. (2012). The best method for isolated total RNA from durian tissues. International Food Research Journal, 19(3): 1181-1183.
9. Wilkins, T. A., Smart, L. B. (1996). Isolation of RNA from Plant Tissue. In: Krieg PA (Editor) *A* Laboratory Guide to RNA: Isolation, Analysis, and Synthesis New York, NY: Wiley-Liss, Inc.
10. Chomczynski, P., Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Annals of Biochemistry, 162: 156-159.
11. Suzuki, Y., Makino, A., Mae, T. (2001). An efficient method for extraction of RNA from rice leaves at different ages using benzyl chloride. Journal of Experimental Botany, 52(360): 1575-9.
12. William, F., Thompson, Marylee, E., Neil, O. P., Richard, A. J., Jeffrey, D. P. (1983). Phytochrome control of RNA levels in developing pea and mung-bean leaves. Planta, 158(6): 487-500.
13. Abbas, A., Makoto, F., Shoji, U., Takashi, Y. (2005). Isolation of high-quality RNA from high-phenolic tissues of eelgrass (*Zostera marina* L.) by keeping temperature low. Plant Molecular Biology Reports, 23(4): 421.

14. Datta, K., Schmiit, A., & Marces, A. (1989). Characterization of two Soybean repetitive proline rich proteins and a cognate cDNA from germinated axes. Plant Cell, 1: 945-952.

15. Kansal, R., Kuhar, K., Verma, I., Gupta, R. N., Gupta, V. K., Koundal, K. R. (2008). Improved and convenient method of RNA isolation from polyphenols and polysaccharide rich plant tissues. Indian Journal of Experimental Biology, 46: 842-845.

16. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.

17. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., Jones, K. M. (1986). Data for Biochemical Research, Oxford Science Publication, 380-381.

18. Venkatachalam, P., Thanseem, I., Thulaseedharan, A. (1999). A rapid and efficient method for isolation of RNA from bark tissues of Heveabrasilienswas. Current Science, 77: 635-637.

19. Tao, Wang., Nianhui, Z., Lingfang, Du. (2005). Isolation of RNA of high quality and yield from *Ginkgo biloba* leaves. Biotechnology Letters, 27(9): 629-633.

20. Salzman, R. A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M., Bressan, R. A. (1999). An Improved RNA Isolation Method for Plant Tissues Containing High Levels of Phenolic Compounds or Carbohydrates. Plant Molecular Biology Reports, 17(1): 11-17.

21. Barlow, J. J., Mathias, A.P., Williamson, R., Gammack, D. B. (1963). A Simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. Biochemistry. Biophysics Research Communication, 13: 61-66.

22. Cathala, G., Savouret, J. F., Mendez, B., West, B. L., Karin, M., Martial, J. A., & Baxter, J. D. (1983). DNA, 2: 327-333.

23. Hidayah, J., (2013). Isolation of high quality RNA from plant rich in flavonoids, *Melastoma decemfidum* Roxb ex. Jack. Australian Journal of Crop Science, 7(7): 911-916.